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tox (TNF)<sup>22,34-41</sup>. This factor causes necrosis  $\lambda$  in  $\lambda$  cells; it is also active in causing lysis of murine L-929 cells  $\lambda$  in  $\lambda$  vitro<sup>22,39</sup>. TNF activity on L-929 cells is not inhibited by neutralizing antibodies specific for lymphotoxin<sup>13</sup>, however, and appears to have distinct biochemical properties<sup>39,40</sup>. Furthermore, the lymphotoxin cDNA probe failed to hybridize on Northern blots to mRNA from induced macrophages producing lytic activity (Fig. 2). The isolation of a TNF DNA cDNA either demonstrates that lymphotoxin and TNF are distinct molecules (see accompanying article<sup>42</sup>).

A cytolytic factor derived from B-cell lines which displays  $\lambda$  in  $\lambda$  and  $\lambda$  in  $\lambda$  vivo anticellular activity has been described<sup>43</sup>; this has been designated 'tumour necrosis factor' based on its activity in the MethA sarcoma assay. This activity probably results from a phototoxin, however, because it has similar biological properties, biochemical properties and is made by the cell line PMI-1788 used in this study for the purification of lymphotoxin. Natural killer cells also can be induced to secrete an anticellular factor<sup>44,45</sup>. The lymphotoxin gene probe and lymphotoxin-specific antibodies will be useful in determining the

Received 20 August; accepted 9 November 1984.

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relationship of this natural killer cell cytotoxic factor to lymphotoxin.

Lymphotoxin has been reported to act synergistically with  $\alpha$ -interferon<sup>46</sup> and  $\gamma$ -interferon<sup>13,14,47</sup>  $\lambda$  in  $\lambda$  vitro and  $\lambda$  in  $\lambda$  vivo. The potent antitumour activity of  $\gamma$ -interferon + lymphotoxin in natural preparations may be a result of the synergistic activity when both lymphokines are present<sup>13</sup>. The ready availability of lymphotoxin produced via recombinant methods will aid the biological characterization of this anticellular lymphokine. It will also help to define the antitumour mechanism of lymphotoxin, as well as its role  $\lambda$  in  $\lambda$  vivo in the regulation of the immune system and its interaction with other lymphokines.

We thank William Kohr and Rod Keck for protein sequencing studies; Dr Mark Matteucci for aid with the lymphotoxin synthetic gene design; the DNA Synthesis Group for preparation of oligomers; Dr Peter Seeburg for the  $\lambda$  gt10 vector; Dr David Goeddel for helpful suggestions, direction, and critical review of this manuscript; Irene Figari and Refaat Shalaby for tumour necrosis assays and the Bioassay Group for performing the murine L-929 assays.

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## Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin

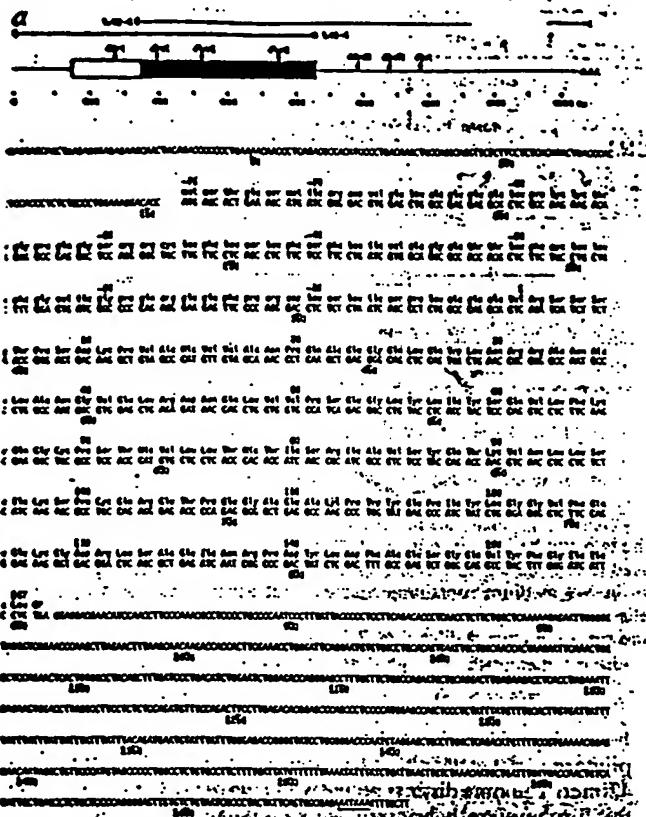
Jane Pennica, Glenn E. Nedwin, Joel S. Hayflick, Peter H. Seeburg, Rik Deryck, Michael A. Palladino\*, William J. Kohr†, Bharat B. Aggarwal\* & David V. Goeddel

Departments of Molecular Biology, \* Pharmacological Sciences and † Protein Biochemistry, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

Human tumour necrosis factor has about 30% homology in its amino acid sequence with lymphotoxin, a lymphokine that has similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary DNA in *Escherichia coli* and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas in syngeneic mice.

TUMOUR necrosis factor (TNF) has been associated with  $\lambda$  in  $\lambda$  and  $\lambda$  in  $\lambda$  killing of tumour cells. This activity was discovered originally in the sera of mice and rabbits injected  $\lambda$  with *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG) or other immunostimulatory agents, and subsequently

with endotoxin<sup>1,2</sup>. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice<sup>1,2</sup>. TNF-like activity has also been detected in the media of BCG/endotoxin-induced monocyte cultures (reviewed in ref. 2) and mitogen-stimulated peripheral



**Fig. 1** TNF cDNA sequences and predicted amino acid sequence. **a**, Schematic representation of human TNF cDNA clones. Overlapping clones A42-4 and A16-4 used in sequence determination and a schematic diagram of the complete cDNA structure are shown. Line, untranslated sequence; boxes, coding sequences; white portion, sequences encoding the signal peptide; shaded regions code for mature TNF. The black box on the 3' end of clone A16-4 indicates that this clone was obtained by specific priming. **b**, Nucleotide sequence and deduced amino acid sequence of human TNF cDNA. Numbers above each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The amino acid labelled 1 represents the first amino acid of mature TNF<sup>14</sup>. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the poly-A<sup>14</sup> adenylation recognition site. **c**, Methods: **a**, Total RNA was extracted<sup>20</sup> from HL-60 cultures 4 h after PMA induction and poly(A)-containing RNA was purified on oligo(dT)-cellulose<sup>21</sup>. Double-stranded cDNA was prepared by oligo(dT) priming<sup>22</sup> using 7.5  $\mu$ g mRNA and fractionated on a 6% polyacrylamide gel. 700 ng cDNA >600 bp was recovered by electroelution. Synthetic EcoRI adaptors<sup>23</sup> were ligated to 20 ng cDNA<sup>24</sup> before ligating into *λ*gt10 (ref. 26). 200,000 cDNA clones were obtained. The same conditions were used to prepare a specifically primed cDNA library of 200,000 clones using as primer the hexadecanucleotide dTGGATGTTCCGTCCTCC (complementary to nucleotides 855-870). Plaque screening<sup>25</sup>, <sup>32</sup>P-radiolabelling of synthetic 42-mer probe<sup>26</sup> and hybridizations<sup>27</sup> were performed. DNA sequencing was performed by the dideoxynucleotide chain termination procedure<sup>28-31</sup>. The cDNA insert of A42-4 consists of nucleotides 337-1643 and the cDNA insert of A16-4 consists of nucleotides 1-870.

blood leukocytes (PBLs)<sup>2</sup>.

TNF activity is cytolytic or cytostatic against many transformed cell lines *in vitro* without obvious species specificity, yet has no known effect on normal mouse embryo fibroblasts or non-transformed cell lines<sup>1,2,4-6</sup>. Activated macrophages may constitute the major cellular origin of TNF<sup>1,3,9,10</sup>, providing an important criterion for distinguishing this factor from the lymphoid cell-derived cytotoxin, lymphotoxin<sup>11</sup>. The primary structure of lymphotoxin was determined recently by protein sequencing<sup>12</sup> and complementary DNA cloning (see accompanying article<sup>13</sup>).

**Table 1** Human TNF production by cell populations and cell lines

Cell source	Inducing agent(s)	Cytotoxic activity (U ml <sup>-1</sup> )	
		TNF	Lymphotoxin
Unfractionated PBLs	None	<8	<8
	LPS	20	<8
	BCG	82	<8
	BCG/LPS	86	<8
	BCG/LPS/PMA	140	<8
	PMA	280	<8
	SEB/T $\alpha_1$	100	10
	SEB/T $\alpha_1$ /PMA	1,600	200
PBLs (adherent cells)	None	<8	<8
	BCG/LPS	350	<8
	SEB/T $\alpha_1$ /PMA	590	<8
PBLs (non-adherent cells)	None	<8	<8
	BCG/LPS	<8	<8
	SEB/T $\alpha_1$ /PMA	<8	350
HL-60	None	<8	<8
	PMA	380	<8
U-937	None	<8	<8
	PMA	32	<8

PBLs were obtained from plateletpheresis residues (Boston Red Cross) by Ficoll-Hypaque centrifugation<sup>9</sup>. Separation of PBLs into adherent and non-adherent populations was performed as described previously<sup>11</sup>. HL-60 (CCL 240) and U-937 cell lines (CRL 1593) were obtained from the American Type Culture Collection. Cells were suspended at  $5 \times 10^6$  cells ml<sup>-1</sup> in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents:  $2 \times 10^5$  organisms per ml of BCG (Calbiochem-Behring), 20  $\mu$ g ml<sup>-1</sup> *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma), 1  $\mu$ g ml<sup>-1</sup> staphylococcal enterotoxin B (SEB, Sigma), 1  $\mu$ g ml<sup>-1</sup> thymosin  $\alpha_1$  (T $\alpha_1$ )<sup>12</sup> and 10 ng ml<sup>-1</sup> PMA (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/PMA treatments; for these two inductions a 24-h BCG stimulation was followed by an additional 24-h treatment with LPS and LPS/PMA, respectively. Samples were assayed for cytolytic activity on mouse L-929 fibroblasts as described previously<sup>11</sup>. The activities shown represent TNF-specific or lymphotoxin-specific units as determined after antibody neutralization at 4 °C for 4 h before assay. The units indicated were obtained from one representative donor in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (L. Svedersky and T. Bringman, unpublished results). Rabbit anti-human lymphotoxin antiserum was prepared against purified human lymphotoxin from RPMI 1788 lymphoblastoid cells<sup>11</sup>.

Here we identify a cell line with monocyte-like characteristics providing a source for human TNF and its messenger RNA. cDNA clones were isolated that encode a polypeptide related structurally to lymphotoxin. This cDNA was engineered to direct the synthesis of a relative molecular mass ( $M_r$ ) 17,000 protein in *E. coli* with the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

### A human TNF-producing cell line

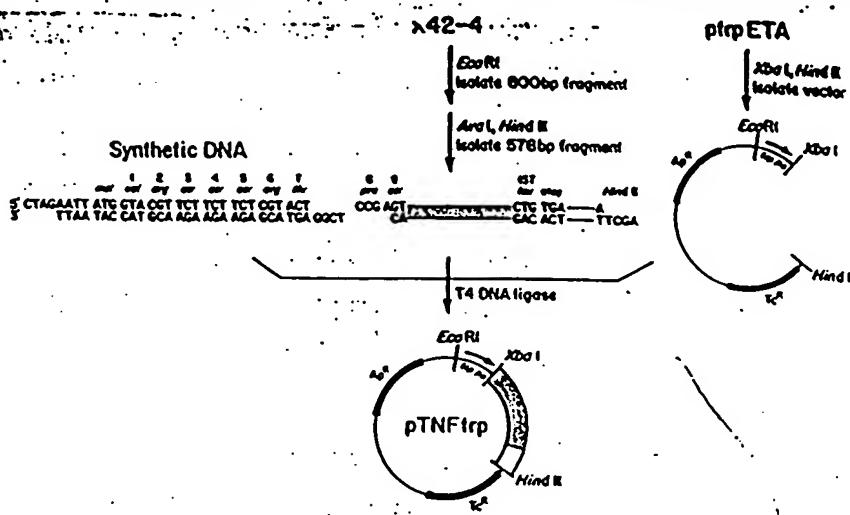
We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent monocytic and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table 1). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphotoxin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphotoxin. Moreover, the results of previous *in vivo* studies using BCG/LPS induction procedures<sup>1,2</sup> demonstrate that the activity can probably be attributed to TNF. Antiserum raised against partially purified PBL-produced TNF completely neutralized this activity (Table 1).

Fig. 1. Construction of a plasmid coding for the direct expression of mature human TNF in *E. coli*. The recombinant phage  $\lambda$ 42-4 (10  $\mu$ g) was digested with *Eco*RI and the 800-bp fragment containing the entire TNF coding region was isolated. TNF digestion with *Xba*I and *Hind*III gave a 578-bp fragment coding for amino acids 8-157. Two synthetic complementary deoxyoligonucleotides<sup>11</sup>, 5'-dCTAGAAT-TATGGTACGGTTCTCTCTCTGACT, and 5'-dTCGGAGTACGAGAAGAACGTA-CCATAAT, were designed to code for amino acids 1-7 of TNF, preceded by an ATG translational initiation codon, and to contain an *Xba*I cohesive terminus. The choice of codons for the first six amino acids of TNF was based on *E. coli* codon usage preferences<sup>12</sup>. An AATT sequence was incorporated upstream of the ATG to maximize expression by giving optimal spacing between the initiation codon and the *up* leader Shine-Dalgarno sequence<sup>13</sup>. The pBR322-derived plasmid pTRpETA<sup>11</sup> was cleaved with *Hind*III and *Xba*I and the large fragment recovered by electroelution. The *Xba*I-*Hind*III fragment and the two synthetic deoxyoligonucleotides were inserted into the plasmid pTNFtrp expression vector to give the plasmid pTNFtrp. The methods used to assemble the fragments and verify the construction of pTNFtrp have been described previously<sup>11,20</sup>. *E. coli* W3110/pTNFtrp was grown in M9 medium containing 5  $\mu$ g ml<sup>-1</sup> tetracycline to 0.2  $A_{550}$  units. Indole acetic acid was added to a concentration of 20  $\mu$ g ml<sup>-1</sup>. The cells were collected at  $A_{550} = 1.0$  and washed with cold PBS. The final cell pellet was resuspended in 1 ml PBS, sonicated on ice for 30 s and the resulting extract diluted in PBS for assay on L-929 cells<sup>11</sup>.

Yields of adherent cells from peripheral blood were low and levels of TNF produced were variable and donor-dependent; therefore tested alternative induction schemes for the production of TNF from total PBLs (Table 1). An increase in cytotoxicity was observed when the PBLs were co-stimulated with *Escherichia coli* enterotoxin B, desacetyl-thymosin- $\alpha_1$  and the mitogen-promoting agent 4 $\beta$ -phorbol 12 $\beta$ -myristate 13 $\alpha$ -acetate (PMA). However, antibody neutralization experiments demonstrated that a significant portion of measured activity was lymphotoxin. Therefore, we screened a number of transformed cell lines of haematopoietic origin for their ability to synthesize TNF. Activity which could be neutralized by anti-TNF antibody was detected following PMA treatment in two monocytic cell lines, HL-60, derived from a promyelocytic leukaemia<sup>14</sup>, and U-937, derived from a histiocytic lymphoma<sup>15</sup> (Table 1). The HL-60 cell line consistently produced higher TNF titres (0-400 U ml<sup>-1</sup> 24 h after induction) than the U-937 cell line (100 U ml<sup>-1</sup>). A time course of TNF synthesis by HL-60 cultures indicated that measurable activity was detected 2 h after PMA treatment (data not shown). Therefore the HL-60 cell line was selected for future experiments; supernatants were collected 24 h after induction for protein purification and 4-h inductions were used when cells were collected for RNA isolation.

#### TNF cDNA clone identification

Human TNF was purified to homogeneity from filtrates of PMA-stimulated HL-60 cell cultures (see ref. 16). A single monomer of  $M_r$  17,000 was observed when the purified TNF was analysed by SDS-gel electrophoresis in reducing conditions. To obtain amino acid sequence information, tryptic peptides of TNF were prepared and separated by reverse-phase HPLC. The preliminary sequence Glu-Thr-Pro-Glu-Gly-Ala-Glu-a-Lys-Pro-Trp-Tyr-Glu-Lys was determined for the first tryptic fragment (TD-6) analysed. A single synthetic 42-base long deoxyoligonucleotide (42-mer) which could code for this amino acid sequence was chemically synthesized<sup>17</sup> for use as a hybridization probe. The design of the probe sequence (dGAAACCCCT-AAGGCGCTAACCCAAACCCCTGGTATGAAAAG) was based on published human codon usage frequencies<sup>18</sup> and the codon bias of human  $\gamma$ -interferon<sup>19</sup>, tissue-type plasminogen activator<sup>20</sup> and lymphotoxin<sup>21</sup>. The general usefulness of this 'probe' approach has been demonstrated recently by the identification of several cloned genomic DNA sequences<sup>21-23</sup> and cDNAs<sup>24,25</sup>.



An oligo(dT)-primed HL-60 cDNA library of ~200,000 clones prepared in  $\lambda$ gt10 (ref. 26) was screened with the <sup>32</sup>P-labelled 42-mer. The nine distinct phage which gave positive signals with this probe were hybridized with 'induced' and 'non-induced' <sup>32</sup>P-labelled cDNA probes<sup>19</sup> prepared using poly(A) mRNA obtained from 4-h PMA-treated and untreated HL-60 cultures, respectively. Seven of these recombinant phage DNAs hybridized weakly to the induced probe but did not hybridize to the uninduced probe, as expected for authentic TNF cDNAs. Restriction endonuclease mapping indicated that these seven cDNA clones were related to each other and that the phage  $\lambda$ 42-4 contained the longest cDNA insert.

#### TNF cDNA sequence

We determined the sequence of the 1,300 base pair (bp) cDNA insert of phage  $\lambda$ 42-4 (nucleotides 337-1,643; Fig. 1). Alignment of the cDNA sequence with the 42-mer probe sequence gave the proper reading frame of the cDNA and demonstrated that it did indeed encode TNF. Of the 14 amino acids (residues 104-117, Fig. 1) assigned to tryptic peptide TD-6 on the basis of preliminary protein sequence, 13 were correct; the only discrepancy was in the last amino acid (position 117) where the cDNA sequence encodes a proline residue rather than the predicted lysine. Despite this difference, the hybridization of the synthetic probe to the TNF cDNA clone was successful, as the 42-mer matched the cDNA sequence in 34 of the first 38 positions, including a stretch of 17 consecutive homologous nucleotides (nucleotides 711-727; Fig. 1).

The assignment of valine (residue 1, Fig. 1) as the first residue of mature TNF was based on NH<sub>2</sub>-terminal protein sequence analysis of the intact molecule (Val-Arg-Ser-Ser-Ser-...)<sup>16</sup>. There are 156 amino acids encoded after this valine before an in-phase termination codon occurs. The coding region of TNF is followed by 792 nucleotides of 3' untranslated sequence containing the hexanucleotide AATAAA (position 1,630-1,635) which precedes the site of polyadenylation in most eukaryotic mRNAs<sup>27</sup>.

Additional confirmation that this sequence codes for TNF was obtained by determining the amino acid sequence of nine tryptic peptides of natural HL-60 TNF and several peptides generated by digesting it with *S. aureus* V8 protease and chymotrypsin<sup>16</sup>. The  $M_r$  of 17,356 calculated for the mature TNF monomer from the cDNA sequence agrees closely with the value obtained for natural TNF by SDS-polyacrylamide gel electrophoresis and amino acid composition<sup>16</sup>. These results and

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Table 2 Necrosis of MethA sarcoma *in vivo*

Treatment	NF			Necrotic response
	++	++	+	
	++	++	+	No. of mice
	0	1	1	9
PBS, i.l.	0	0	1	4
PBS, i.p.	0	0	1	4
PBS, i.m.	0	0	1	4
<i>E. coli</i> LPS, i.l.	0	0	1	9
HL-60 TNF, i.l.	5	5	0	0
rTNF, i.l.	7	5	0	0
rTNF, i.p.	2	1	0	2
rTNF, i.m.	2	2	1	0

(BALB/c x C57BL/6)F<sub>1</sub> female mice were injected intradermally with  $5 \times 10^5$  BALB/c MethA sarcoma cells. Ten days later, the tumours (0.75 cm average diameter) were injected intralesionally (i.l.,  $1 \times 10^5$  U), intraperitoneally (i.p.,  $5 \times 10^5$  U) or intramuscularly (i.m.,  $5 \times 10^5$  U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment the tumours were excised, sectioned and scored for haemorrhagic necrosis by visual and histological examination as described previously<sup>1</sup>. In the maximum response (++) 50–75% of the tumour mass is markedly necrotic after 24 h; + denotes a moderate response, that is 25–50% haemorrhagic necrosis; +, a minimal response of <25% haemorrhagic necrosis; –, tumours showed no visible necrosis. Natural TNF was purified from HL-60 cultures as described elsewhere<sup>16</sup>. Recombinant TNF (rTNF) was purified from *E. coli* W3110/pTNF<sup>trp</sup> to a purity of >95% and a specific activity of  $\sim 10^6$  units/mg (T. Bringman, unpublished results).

The absence of any potential *N*-glycosylation sites in the deduced amino acid sequence suggest that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multi-molecular form, as the *M<sub>r</sub>* estimated previously for human TNF ranged from 34,000–140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are probably involved in a single intramolecular disulphide bond<sup>16</sup>.

The cDNA clone  $\lambda 42-4$  contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-primed cDNA library was prepared (see Fig. 1 legend) and screened with the <sup>32</sup>P-labelled  $\lambda 42-4$  cDNA insert. A cDNA clone ( $\lambda 16-4$ ) was identified which contained an insert extending 337 bp further 5' than the  $\lambda 42-4$  insert (Fig. 1).

From an analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as part of a larger precursor (pre-TNF). Starting at the 5' end of the cDNA, 125 nucleotides of non-translated sequence are followed by a methionine codon and an open reading frame of 233 amino acids. This AUG is preceded by termination codons in all three frames, suggesting that it is the initiation codon. Furthermore, the sequence context of this AUG conforms closely to the CCA<sub>n</sub>AUG proposed<sup>29</sup> as a consensus sequence for eukaryotic initiator sites.

The presequence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF polypeptide and contains an unusually long hydrophobic region of 26 amino acids (residues –46 to –21). Typically, signal peptides involved in protein secretion are only 20–30 amino acids long<sup>30,31</sup>. However, a signal sequence for the Rous sarcoma virus envelope glycoprotein<sup>32</sup> is atypically long (63 residues) and contains also many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules<sup>33–35</sup>.

We used the <sup>32</sup>P-labelled  $\lambda 42-4$  cDNA insert to examine TNF gene structure and mRNA size. Results from Southern<sup>37</sup> hybridizations indicate that only a single gene for TNF is present in the human genome. Northern hybridization analysis<sup>38</sup> shows that a single mRNA species  $\sim 18S$  in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated from human PBMs. This provides additional evidence that the same cytokine is produced from both

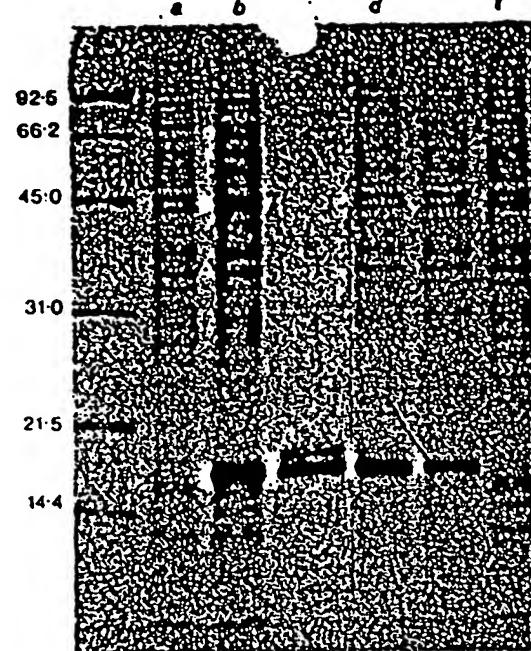


Fig. 3 SDS-polyacrylamide gel electrophoresis of human TNF synthesized in *E. coli*. *E. coli* K-12 strain W3110, transformed with pTNF<sup>trp</sup> or pBR322, was grown in M9 medium containing  $5 \mu\text{g ml}^{-1}$  tetracycline. Cells were collected, lysed in 2% SDS, 1%  $\beta$ -mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maizels<sup>32</sup> and the gel stained with Coomassie brilliant blue. The left lane contains protein *M<sub>r</sub>* standards ( $\times 10^{-3}$ ): phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes a, f, cell lysates of *E. coli* W3110/pBR322; lanes b, e, cell lysates of *E. coli* W3110/pTNF<sup>trp</sup>; lane c, partially purified human TNF isolated from the HL60 cell line<sup>16</sup>; lane d, mixture of the *E. coli* W3110/pTNF<sup>trp</sup> cell lysate and the HL60-derived, purified TNF.

cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

#### TNF synthesis in *E. coli*

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in *E. coli* (Fig. 2). In the resulting expression plasmid, pTNF<sup>trp</sup>, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the *E. coli* *trp* operon containing the *trp* promoter, operator and Shine-Dalgarno sequence of the *trp* leader peptide.

Total extracts of *E. coli* K-12 strain W3110 transformed with pTNF<sup>trp</sup> contained a prominent polypeptide with an apparent *M<sub>r</sub>* 17,000 (Fig. 3, lanes b, e). This protein is not visible in cells transformed with pBR322 (lanes a, f), strong evidence that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane c) isolated from the HL-60 cell line (lane d), suggesting that no significant post-translational processing of TNF occurs in the HL-60 cell line. This is unlike lymph toxin and  $\gamma$ -interferon, both of which occur naturally as heterogeneous glycoproteins as a consequence of N-terminal<sup>12</sup> and C-terminal<sup>39</sup> proteolysis, respectively.

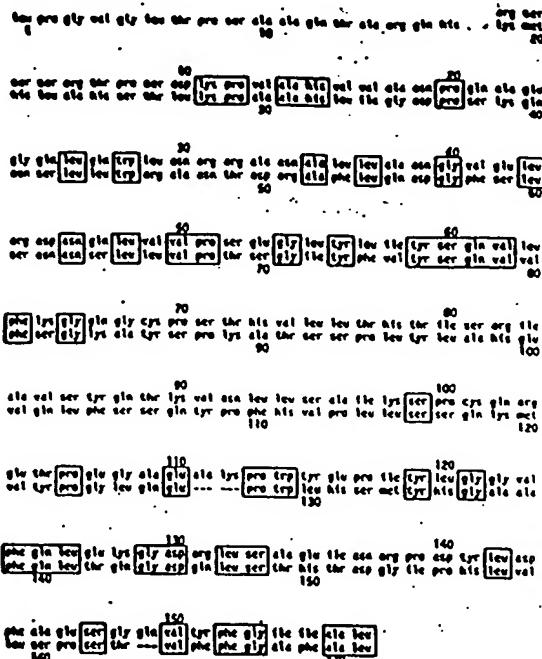


Fig. 4 Comparison of the amino acid sequence of human TNF with human lymphotoxin<sup>12,13</sup>. The sequences have been aligned to give maximal homology by introducing two gaps (indicated by dashed lines) into the lymphotoxin sequence. Identical amino acids are boxed. The numbers above each row (1-157) and below each row (1-171) indicate the amino acids of mature TNF and lymphotoxin (LT), respectively.

Verification of the bacterial production of biologically-active IF was obtained by assaying extracts of *E. coli* W3110/pTNFtrp for cytolytic activity in the murine L-929 fibroblast assay<sup>11</sup>. Approximately 300,000 units of activity were detected per ml of culture at  $A_{550} = 1$ , whereas no activity was observed in *E. coli* W3110/pBR322 controls. This corresponds to  $\sim 3$  ng of active TNF per 1 ( $A_{550} = 1$ ) or about 300,000 picoles of active TNF per cell if a specific activity of  $10^6$  picos/mg<sup>-1</sup> (ref. 16) is assumed. The activity was neutralized by serum prepared against partially purified PBL-derived TNF, but was not neutralized by preimmune serum or rabbit anti-human lymphotoxin antibodies (data not shown).

### *In vivo* necrosis activity

IF is generally defined as a cytotoxin released by BCG/LPS-activated macrophages which induces the haemorrhagic necrosis of methylcholanthrene-induced (MethA) sarcomas in BALB/c mice<sup>14</sup>. Therefore, we examined recombinant human TNF produced from *E. coli* and natural human TNF from PMA-activated HL-60 cultures for *in vivo* tumour necrosis activity in MethA assay<sup>1</sup>. Both recombinant and natural TNF samples showed significant necrotic responses, regardless of whether the IF was injected intralesionally or systemically (Table 2). Tumour necrosis of the MethA sarcoma tumours was observed in mice injected with either phosphate-buffered saline (PBS) or 100 µg *E. coli* LPS. These results, taken with the antibody neutralization and Northern hybridization data, provide further evidence that the cytotoxin described here is human IF.

### Homology to lymphotoxin

Known *in vivo* and *in vitro* biological activities of TNF and lymphotoxin are very similar<sup>2,3,13</sup>. TNF and lymphotoxin are known to be antigenically distinct molecules. It has thus some common to distinguish these two lymphokines on the basis of the cell populations responsible for their synthesis. We compared the amino acid sequences of human TNF and

lymphotoxin to determine whether similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphotoxin sequence can be aligned with the TNF sequence so that distinct homologies are apparent; we find 44 of the 157 TNF residues (28%) are identical to corresponding lymphotoxin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35-66 and 110-133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphotoxin. The hydrophobic carboxy-termini of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytotoxic activities of TNF and lymphotoxin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytotoxic activity in a truncated lymphotoxin polypeptide lacking its last 16 amino acids<sup>13</sup>.

Lymphotoxin has 18 more NH<sub>2</sub>-terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytotoxic activity. In fact, a 148 residue lymphotoxin, consisting of amino acids 24-171 of mature lymphotoxin, and having similar cytotoxic effects on L-929 cells, has been isolated from the RPMI-1788 cell line<sup>11,12</sup>. It is also interesting that amino acids 67-109 of TNF are unrelated to the corresponding region of lymphotoxin; only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys 69-Cys 101 disulphide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytotoxic activity. Such positioning, which could be achieved by a TNF disulphide bond, may require a very different sequence of amino acids in lymphotoxin, where no disulphide bond exists. These apparently unrelated regions of TNF and lymphotoxin might specify also as yet undiscovered differences in biological activities and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphotoxin<sup>13</sup>, in combination with the techniques of site-directed mutagenesis<sup>10</sup>, will make it possible to address questions of this type directly.

We thank Phil Hass for growing HL-60 cells; Dr Lloyd Svedersky and Tim Bringman for preparing TNF antiserum; Mark Vasset, Parkash Jhurani and Peter Ng for deoxyoligonucleotide synthesis; Irene Figari and Refaat Shalaby for assistance with the tumour necrosis assays; Roxanne Chang and the Genentech Bioassay Group for performing *in vitro* TNF assays; and Dr Richard Harkins for helpful suggestions. G.E.N. dedicates this work to the late Jack L. Levenson.

Received 20 August; accepted 9 November 1984.

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32. Noda, T.
33. Oishi, T.
34. Amano, T.
35. Saito, T.

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# Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

Hiroyuki Nawa, Hirokazu Kotani & Shigetada Nakanishi

Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto 606, Japan

A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance P alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.

SUBSTANCE P is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurones<sup>1</sup>. Substance P belongs to a family of related peptides, the tachykinins, and is thought to be the only member of this family present in mammalian tissues<sup>2</sup>. Recently, we elucidated the entire primary structures of two types of bovine brain substance P precursors ( $\alpha$ - and  $\beta$ -preprotachykinins) by determining their cloned cDNA sequences<sup>3</sup>.  $\beta$ -Preprotachykinin ( $\beta$ -PPT) contains not only the substance P sequence but also a novel tachykinin sequence designated substance K, whereas  $\alpha$ -preprotachykinin ( $\alpha$ -PPT) lacks the latter sequence, containing only substance P. The decapeptide substance K has been found independently as neurokinin  $\alpha$ , a gut-contracting peptide in porcine spinal cord<sup>4</sup>. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinin family, but is considerably more potent than substance P in some pharmacological tests<sup>5,6</sup>. Substance K thus represents a second type of mammalian tachykinin which may have a physiological role different from that of substance P in mammalian organisms.

The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5' and 3' sequences and differ only in the insertion/deletion of the sequence coding for the substance K region<sup>3</sup>. This characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the PPT gene and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both  $\alpha$ - and  $\beta$ -PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

## PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization

*in situ* with a bovine  $\beta$ -PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kbp) length of a continuous genomic DNA (Fig. 1a; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1 b-f). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1g). Intron A (403 base pairs, bp) is inserted within the segment encoding the 5'-untranslated region of the mRNA, 9-10 bp upstream from the translational initiation site. Introns B (~1.0 kbp), C (~450 bp), D (~460 bp), E (~1.4 kbp) and F (~3.6 kbp) all interrupt the protein-encoding region of the gene. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes<sup>7</sup>. Exons 2-7 consist of 132, 97, 45, 24, 54 and 596 bp, each encoding the protein sequence corresponding to the signal peptide, substance P, two spacer sequences, substance K, and the C-terminal sequence, respectively. It is remarkable that exon 6 precisely specifies the substance K region missing in  $\alpha$ -PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both  $\alpha$ - and  $\beta$ -PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5' termini of the PPT mRNAs were identified by S<sub>1</sub> nuclease mapping and primer extension analyses (Fig. 2). Both analyses revealed a length heterogeneity at the 5' end of the PPT transcripts. The major 5' termini of the PPT mRNAs mapped at 106-108, 110 and 111 bp upstream from the 3' end of exon 1 (Fig. 1g). Several minor mRNA species starting further upstream were also observed and these 5' termini mapped at roughly 132, 133, 137 and 146 bp upstream from the 3' end of exon 1. In support of these assignments, we found that three of the four cDNA clones isolated previously<sup>3</sup> (clones pSP301, 302 and 306) contained the extreme 5' sequences corresponding to the major 5' ends, while the remaining one (clone pSP307) extended its 5'-terminus up to one of the minor 5' ends. Based on the assignments of the 5' termini of the PPT mRNAs, we conclude that the bovine PPT gene is ~8.4 kbp long.


*Protein*

PubMed	Nucleotide	Protein	Genome	Structure	PopSel	Taxonomy	OMIM
Search <input checked="" type="checkbox"/> Protein for <input checked="" type="checkbox"/>				<input type="button" value="Go"/> <input type="button" value="Clear"/>			
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Γ 1: QWHUN tumor necrosis factor alpha precursor - human

BLINK, PubMed, Related Sequences, Taxonomy, OMIM

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**VERSION** QWHUN GI:69405  
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genetic: #gene GDB:TNF; TNFA ##cross-references GDB:120441;  
OMIM:191160 #map\_position 6p21.3-6p21.3 #introns 62/3; 78/1; 94/1  
**COMPLEX** homotrimer;  
superfamily: tumor necrosis factor;  
PIR dates: 28-Aug-1985 #sequence\_revision 28-Aug-1985 #text\_change  
04-Feb-2000.  
**KEYWORDS** cytokine; cytotoxin; glycoprotein; homotrimer; lipoprotein;  
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**SOURCE** human:  
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Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.  
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**AUTHORS** Pennica,D., Nedwin,G.E., Hayflick,J.S., Seeburg,P.H., Deryck,R.,  
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**JOURNAL** Nature 312 (5996), 724-729 (1984)  
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**REFERENCE** 2 (residues 1 to 233)  
**AUTHORS** Aggarwal,B.B., Kohr,W.J., Hass,P.E., Moffat,B., Spencer,S.A.,  
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**JOURNAL** J. Biol. Chem. 260 (4), 2345-2354 (1985)  
**MEDLINE** 85130974  
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**AUTHORS** Wang,A.M., Creasey,A.A., Ladner,M.B., Lin,L.S., Strickler,J., Van  
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**JOURNAL** Science 228 (4696), 149-154 (1985)  
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**REFERENCE** 4 (residues 1 to 233)  
**AUTHORS** Nedwin,G.E., Naylor,S.L., Sakaguchi,A.Y., Smith,D.,  
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**JOURNAL** Nucleic Acids Res. 13 (17), 6361-6373 (1985)  
**MEDLINE** 8604093

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**AUTHORS** Marmenout, A., Fransen, L., Tavernier, J., Van der Heyden, J.,  
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**TITLE** Molecular cloning and expression of human tumor necrosis factor and  
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**JOURNAL** Eur. J. Biochem. 152 (3), 515-522 (1985)  
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**AUTHORS** Fukuda, S., Ando, S., Sanou, O., Taniai, M., Fujii, M., Masaki, N.,  
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**JOURNAL** Lymphokine Res. 7 (2), 175-185 (1988)  
**MEDLINE** 88301617  
**REFERENCE** 7 (residues 1 to 233)  
**AUTHORS** Stevenson, F.T., Bursten, S.L., Locksley, R.M. and Lovett, D.H.  
**TITLE** Myristyl acylation of the tumor necrosis factor alpha precursor on  
 specific lysine residues  
**JOURNAL** J. Exp. Med. 176 (4), 1053-1062 (1992)  
**MEDLINE** 93018820  
**REMARK** annotation; identification of myristylated lysines  
**REFERENCE** 8 (residues 1 to 233)  
**AUTHORS** Iris, F.J.M., Bougueret, L., Prieur, S., Caterina, D., Primas,  
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**TITLE** Dense Alu clustering and a potential new member of the NFkappaB  
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**REFERENCE** 9 (residues 1 to 233)  
**AUTHORS** D'Alfonso, S. and Richiardi, P.M.  
**TITLE** A polymorphic variation in a putative regulation box of the TNFA  
 promoter region  
**JOURNAL** Immunogenetics 39 (2), 150-154 (1994).  
**MEDLINE** 94102809  
**REFERENCE** 10 (residues 1 to 233)  
**AUTHORS** Takakura-Yamamoto, R., Yamamoto, S., Fukuda, S. and Kurimoto, M.  
**TITLE** O-glycosylated species of natural human tumor-necrosis factor-alpha  
**JOURNAL** Eur. J. Biochem. 235 (1-2), 431-437 (1996)  
**MEDLINE** 96202967  
**COMMENT** Secreted from mitogen-activated macrophages within 4-24 hours after  
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 lines and have an antiproliferative effect on others without  
 detriment to normal cells. It can also act synergistically with  
 interferon gamma to kill certain transformed cell lines.  
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  61 eefprdlslsli splaqavrss srtpsdkpvva hvvanpqaeg qlqwl1nrran allangvelr
  121 dnqlvvpsseg lyliysqvlf kgqgcpstthv llthtisria vsyqtkvnll saikspcqr
  181 tpegaeakpw yepiylggvf qlekgdrlsa einrpdylf aesaqqvfygi ial
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```

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REMARK SEQUENCE FROM N.A.  
 REFERENCE 4 (residues 1 to 233)  
 AUTHORS Nedwin, G.E., Naylor, S.L., Sakaguchi, A.Y., Smith, D.,  
 Jarrett-Nedwin, J., Pennica, D., Goeddel, D.V. and Gray, P.W.  
 TITLE Human lymphotaxin and tumor necrosis factor genes: structure,  
 homology and chromosomal localization  
 JOURNAL Nucleic Acids Res. 13 (17), 6361-6373 (1985)  
 MEDLINE 86016093  
 REMARK SEQUENCE FROM N.A.  
 REFERENCE 5 (residues 1 to 233)  
 AUTHORS Wang, A.M., Creasey, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van  
 Arsdell, J.N., Yamamoto, R. and Mark, D.F.  
 TITLE Molecular cloning of the complementary DNA for human tumor necrosis  
 factor  
 JOURNAL Science 228 (4696), 149-154 (1985)  
 MEDLINE 85142190  
 REMARK SEQUENCE FROM N.A.  
 REFERENCE 6 (residues 1 to 233)  
 AUTHORS Marmenout, A., Fransen, L., Tavernier, J., van der Heyde, H.J.,  
 Tizard, R., Kawashima, E., Shaw, A., Johnson, M.J., Semon, D.,  
 Mueller, R., Ruysschaert, M.R., van Vliet, A. and Fiers, W.  
 TITLE Molecular cloning and expression of human tumor necrosis factor and  
 comparison with mouse tumor necrosis factor  
 JOURNAL Eur. J. Biochem. 152 (3), 515-522 (1985)  
 MEDLINE 86030296  
 REMARK SEQUENCE FROM N.A.  
 REFERENCE 7 (residues 1 to 233)  
 AUTHORS Iris, F.J.M., Bougueret, L., Prieur, S., Caterina, D., Primas, G.,  
 Perrot, V., Jurka, J., Rodriguez-Tome, P., Claverie, J.-M., Dausset, J.  
 and Cohen, D.  
 TITLE Dense Alu clustering and a potential new member of the NF kappa B  
 family within a 90 kilobase HLA class III segment  
 JOURNAL Nat. Genet. 3 (2), 137-145 (1993)  
 MEDLINE 93272029  
 REMARK SEQUENCE FROM N.A.  
 REFERENCE 8 (residues 1 to 233)  
 AUTHORS Rowen, L., Madan, A., Qin, S., Shaffer, T., James, R., Ratcliffe, A.,  
 Abbasi, N., Dickhoff, R., Loretz, C., Madan, A., Dors, M., Young, J.,  
 Lasky, S. and Hood, L.  
 TITLE Direct Submission  
 JOURNAL Submitted (??-OCT-1999) to the EMBL/GenBank/DDBJ databases  
 REMARK SEQUENCE FROM N.A.  
 REFERENCE 9 (residues 1 to 233)  
 AUTHORS Jones, E.Y., Stuart, D.I. and Walker, N.P.  
 TITLE Structure of tumour necrosis factor  
 JOURNAL Nature 338 (6212), 225-228 (1989)  
 MEDLINE 89159409  
 REMARK X-RAY CRYSTALLOGRAPHY (2.9 ANGSTROMS).  
 REFERENCE 10 (residues 1 to 233)  
 AUTHORS Jones, E.Y., Stuart, D.I. and Walker, N.P.  
 TITLE The structure of tumour necrosis factor--implications for  
 biological function  
 JOURNAL J. Cell Sci. Suppl. 13, 11-18 (1990)  
 MEDLINE 91193276  
 REMARK X-RAY CRYSTALLOGRAPHY (2.9 ANGSTROMS).  
 REFERENCE 11 (residues 1 to 233)  
 AUTHORS Eck, M.J. and Sprang, S.R.  
 TITLE The structure of tumor necrosis factor-alpha at 2.6 A resolution.  
 Implications for receptor binding  
 JOURNAL J. Biol. Chem. 264 (29), 17595-17605 (1989)  
 MEDLINE 90008932  
 REMARK X-RAY CRYSTALLOGRAPHY (2.6 ANGSTROMS).  
 REFERENCE 12 (residues 1 to 233)  
 AUTHORS Reed, C., Fu, Z.Q., Wu, J., Xue, Y.N., Harrison, R.W., Chen, M.J. and

Weber, I.T.  
 TITLE Crystal structure of TNF-alpha mutant R31D with greater affinity  
 for receptor R1 compared with R2  
 JOURNAL Protein Eng. 10 (10), 1101-1107 (1997)  
 MEDLINE 98147459  
 REMARK X-RAY CRYSTALLOGRAPHY (2.3 ANGSTROMS) OF MUTANT ARG-107.  
 REFERENCE 13 (residues 1 to 233)  
 AUTHORS Cha, S.S., Kim, J.S., Cho, H.S., Shin, N.K., Jeong, W., Shin, H.C.,  
 Kim, Y.J., Hahn, J.H. and Oh, B.H.  
 TITLE High resolution crystal structure of a human tumor necrosis  
 factor-alpha mutant with low systemic toxicity  
 JOURNAL J. Biol. Chem. 273 (4), 2153-2160 (1998)  
 MEDLINE 98113178  
 REMARK X-RAY CRYSTALLOGRAPHY (1.8 ANGSTROMS) OF MUTANT M3S.  
 REFERENCE 14 (residues 1 to 233)  
 AUTHORS Van Ostade, X., Tavernier, J., Prange, T. and Fiers, W.  
 TITLE Localization of the active site of human tumour necrosis factor  
 (hTNF) by mutational analysis  
 JOURNAL EMBO J. 10 (4), 827-836 (1991)  
 MEDLINE 91184128  
 REMARK MUTAGENESIS.  
 REFERENCE 15 (residues 1 to 233)  
 AUTHORS Stevenson, F.T., Bursten, S.L., Locksley, R.M. and Lovett, D.H.  
 TITLE Myristyl acylation of the tumor necrosis factor alpha precursor on  
 specific lysine residues  
 JOURNAL J. Exp. Med. 176 (4), 1053-1062 (1992)  
 MEDLINE 93018820  
 REMARK MYRISTOYLATION.  
 COMMENT -----

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 collaboration between the Swiss Institute of Bioinformatics and  
 the EMBL outstation - the European Bioinformatics Institute.  
 The original entry is available from <http://www.expasy.ch/sprot>  
 and <http://www.ebi.ac.uk/sprot>

[FUNCTION] TNF IS MAINLY SECRETED BY MACROPHAGES, IT IS A CYTOKINE  
 WITH A WIDE VARIETY OF FUNCTIONS: IT CAN CAUSE CYTOLYSIS OF CERTAIN  
 TUMOR CELL LINES, IT IS IMPLICATED IN THE INDUCTION OF CACHEXIA, IT  
 IS A POTENT PYROGEN CAUSING FEVER BY DIRECT ACTION OR BY  
 STIMULATION OF INTERLEUKIN 1 SECRETION, IT CAN STIMULATE CELL  
 PROLIFERATION AND INDUCE CELL DIFFERENTIATION UNDER CERTAIN  
 CONDITIONS.

[SUBUNIT] HOMOTRIMER.

[SUBCELLULAR LOCATION] TYPE II MEMBRANE PROTEIN. ALSO EXISTS AS AN  
 EXTRACELLULAR SOLUBLE FORM.

[PTM] THE SOLUBLE FORM DERIVES FROM THE MEMBRANE FORM BY  
 PROTEOLYTIC PROCESSING.

[DISEASE] CACHEXIA ACCOMPANIES A VARIETY OF DISEASES, INCLUDING  
 CANCER AND INFECTION, AND IS CHARACTERIZED BY GENERAL ILL HEALTH  
 AND MALNUTRITION.

[SIMILARITY] BELONGS TO THE TUMOR NECROSIS FACTOR FAMILY.

FEATURES	Location/Qualifiers
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Region	1..76
Protein	/region_name="Propeptide" 1..233 /product="TUMOR NECROSIS FACTOR PRECURSOR"
Site	1..233 19 /site_type="lipid-binding" /note="MYRISTATE."
Site	20



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/region_name="Helical region"
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222
/site_type="mutagenized"
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Region 224..225
/region_name="Hydrogen bonded turn"
Region 226..232
/region_name="Beta-strand region"

ORIGIN
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61 eefprdlslsli splaqavrss srtpsdkpva hvvanpqaeg qlqwlnnrran allangvelr
121 dnqlvvpsseg lyliysqvlf kgqgcpsthv llthtisria vsyqtkvnll saikspcqre
181 tpegaeakpw yepiylggvf qlekgdrlsa einrpdylf aesgqvyfgi ial
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```

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## Peptide Synthesis Calculation Sheet:

Notebook name is "s309".

Notebook file is "s309.NBK".

Target Peptide: length = 22, MW = 2507.037

NH2-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-  
Gln-Thr-Lys-Val-Asn-Leu-Leu-COOH

Resin substitution = 0.100 meq/g

Resin quantity = 3.000 g

Excess amino acid = 4.000 x *Equivalency checked*Peptide Quantity = 0.300 mMoles *23.10.89*Theoretical Yield = 0.752 g *J.Y.*

Starting Resin: Fmoc-Leu-PepSyn-KA

*Different from normal synthesis  
this time I guess*

## Peptide Synthesis Calculation Sheet:

Notebook name is "s323".  
Notebook file is "S323.NBK".

Target Peptide: length = 11, MW = 1238.416

NH<sub>2</sub>-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-COOH

Resin substitution = 0.100 meq/g  
Resin quantity = 4.000 g  
Excess amino acid = 4.000 x

Peptide Quantity = 0.400 mMoles  
Theoretical Yield = 0.495 g

Sequence checked  
21-11-89

Starting Resin: FMOC-Thr-PepSyn-KA

PEPTIDE TECHNOLOGY LTD  
SYNTHESIS NO. 323 14 mg  
H-Tyr-Ile-Ser-Arg-Ile-Ala-Val-Ser-  
Tyr-Gln-Thr-COOH  
STORE BELOW 8°C...NOT FOR HUMAN USE